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(54) Title: HEPATITIS C VIRUS CELL PROPAGATION AND RELATED METHODS

(57) Abstract

The invention provides a composition comprising hepatitis C virus in cell culture supernatant medium at a titer of at least 10^4 genomes per milliliter of cell culture supernatant medium in the absence of components from primate serum or plasma as determined by reverse transcriptase polymerase chain reaction. The titer can also be from about 10^5 to about 10^6 genomes per milliliter of culture medium. A method for propagating hepatitis C virus in cell culture is also provided, comprising the steps of: (a) contacting a suitable uninfected cell culture with hepatitis C virus; (b) incubating the contacted cell culture in cell culture medium under conditions to permit infection of the culture cells by hepatitis C virus; and (c) propagating hepatitis C virus in the infected culture cells under conditions to produce a virus titer of at least 10^4 genomes per milliliter culture medium.

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HEPATITIS C VIRUS CELL PROPAGATION AND RELATED METHODS

BACKGROUND OF THE INVENTION

Viral Hepatitis C (HCV) has been previously described as parenterally transmitted non-A non-B hepatitis, non-B transfusion-associated hepatitis, and post-transfusion non-A non-B hepatitis. Hepatitis C virus infection is characterized by insidious onset with anorexia, vague abdominal discomfort, nausea and vomiting, and progression to jaundice. The severity of HCV infection ranges from inapparent cases to rare fulminating, fatal cases. It is usually less severe in the acute stage, but chronicity is common. Chronic infection may be symptomatic or asymptomatic and may progress to cirrhosis, but more often improves clinically after 2 to 3 years.

Previously, diagnosis depended on the exclusion of hepatitis A, B and delta virus and other causes of liver injury. A serologic test for antibody to the agent has been recently been developed and is being established as a screening test for blood donors. This test for antibody to hepatitis C virus (anti-HCV) is positive in the majority of patients with chronic hepatitis C (Kuo et al. *Science* 244:362-364, 1989). In patients with acute disease, there may be a prolonged interval between exposure to the virus or the onset of illness and detecting of anti-HCV. More recently developed serologic HCV infection tests detect seroconversion earlier than the above test (Mimms et al. *Lancet* 336:1590-1591, 1990).

Background Art

European Patent Application 0-318-216 (hereinafter "the '216 application" states that an aspect of the invention is a "tissue culture grown cell infected with HCV"; that the "availability of probes for HCV ... allows

for development of tissue culture systems"; and that "HCV particles may be isolated from the sera from BB-NANBV infected individuals, or from cell cultures by any of the methods known in the art ...". The '216 application also suggests that methods for culturing flaviviruses known to those skilled in the art and suitable cell lines known to support flavivirus replication may be used to accomplish HCV infection of cell lines. The '216 application provides no data supporting the existence of an HCV cell culture.

PCT Application WO 92/07001 (hereinafter "the '001 application") states that immunoprecipitation and capture of HCV antibody using solubilized labeled virus as provided in the application can be utilized to identify labeled viral antigens produced in cell lines capable of supporting viral replication, and states that this technique can be used to screen various cell lines for the presence of viral replication. The '001 application does not provide data supporting the existence of HCV-propagating cell cultures or specific protocols for obtaining cell cultures of HCV or for specific methods of using such cell cultures.

PCT Application WO 90/10060 (hereinafter "the '060 application") reports the <u>in vitro</u> culture of NANBH virus comprising infected primary chimpanzee hepatocytes and a cell culture medium with specified components. The '060 application reports that the detection of virus associated marker antigen was never found in more than 10% of inoculated primary hepatocytes. In the inoculation experiment using tissue culture medium, medium samples from time points beginning at day 3 through day 31 were pooled and concentrated to be used as the inoculum. Virus was reported present at day 17 after inoculation of the cell culture. The '060 application does not report a long term cell culture HCV propagation system.

European Patent Application No. EP 0 414 475 (hereinafter "the '475 application") recites the preparation of the HCV infected cell lines according to a variety of methods including the cultivation of susceptible cells with concentrated culture fluids from short term primary cultures of cells that are replicating the virus, direct infection of susceptible cells with isolated HCV particles or growing susceptible cells in contact with HCV infected cells. The '475 application also suggests that infected cells from an individual with HCV may be fused with established cells from a cell line to produce a permanent cell line, or that HCV infected blood cells isolated from an individual may be immortalized. The '475 application does not teach a method or show any method to be effective for obtaining long term cell culture propagation of hepatitis C virus.

Jacob et al (J. Infect. Dis. 161:1121-1127, 1990) reports the expression of infectious viral particles by primary chimpanzee hepatocytes. The disclosure for Jacob et al. is described above with reference to the '060 application, which describes and claims the culture of primary hepatocytes infected with HCV.

Schimizu et al. (Proc. Nat. Acad. Sci. (U.S.A.) 89:5477-5481, 1992) describes the inoculation of human T-cell lines (molt-4 and molt-4 Ma) with serum obtained from a chimpanzee infected with chimpanzee passaged HCV. In molt-4 Ma, cells that were harvested 14 days after inoculation, diluted to 2 x 10^5 cells per milliliter with fresh medium, distributed into culture bottles and subcultured for 5 days without changing the medium, both plus and minus strand HCV RNAs had titers of 10^0 genomes per cell pellet by PCR. Using *in situ* hybridization with the ^{35}S labeled HCV plus strand RNA probe on molt-4 cells harvested 7 days after virus inoculation, minus strand HCV RNA was detected in about 1% of the cells. In another

experiment, 7 days after inoculation about 1% of the cells were positive for the virus encoded proteins, HCV core and NS 4 antigens. 18 days after inoculation most virus was lost.

Zignego, et al. (J. Hepat. 15:382-386, 1992) describes the PCR detection of HCV in peripheral blood mononuclear cells obtained from individuals infected with HCV. The presence of HCV RNA negative strands in subpopulations of peripheral blood mononuclear cells, monocytes, and T and B lymphocytes, is suggested by Zignego et al. to show that viral multiplication probably occurs in these cells. However, the negative strand of HCV has been shown to be present in serum as well as infected tissue. Thus, the presence of negative strand HCV RNA cannot be used as an indicator of virus propagation (Fong et al. *J. Clin. Invest.* 88:1058-1060, 1991).

Ozeki, et al. (Int. J. Exp. Path. 73:1-8, 1992) discloses the apparent infection of cultured Chang cells with HCV from liver extracts of individuals with chronic hepatitis. After a 3 day incubation, the Chang cells exposed to the liver extract and cultured on glass slides were contacted with anti-HCV antibody. In 4 out of 8 liver extract infections, more than 10% of the Chang cells stained positive for HCV using immunofluorescence, and in 6 of 8 liver extract infections, more than 20% of the Chang cells were positive for HCV by the biotin-avidin complex method. However, Ozeki et al. also report that the rate of infection of Chang cells is no greater after 6 days of incubation than after 3 days.

None of the above references discloses a cell culture method for the long term propagation of hepatitis C virus to obtain levels of HCV of at least 10^4 genomes per milliliter of cell culture supernatant medium. Thus,

because of the shortcomings of the prior art methods for culturing hepatitis C virus, the need exists for a cell culture method for propagating HCV to titers sufficient for the development of therapeutic and diagnostic products. The present invention meets this need by the cell culture method described herein.

Specifically, no vaccine nor any effective anti-viral treatment for HCV infection currently exists. Furthermore, there is currently no cost effective way to screen potential vaccine reagents. Thus, there is a need for a method for screening compounds for anti-HCV activity and for HCV vaccine activity. The present invention meets these needs as described herein.

In addition, no plasma or serum component-free composition of HCV at a titer of at least 10^4 genomes per milliliter is available. Such a composition is necessary for the efficient conduct of the above-described anti-HCV and HCV vaccine screening methods. The present invention provides such a composition according to the description below.

SUMMARY OF THE INVENTION

The invention provides a composition comprising hepatitis C virus in cell culture supernatant medium at a titer of at least 10^4 genomes per milliliter of cell culture supernatant medium in the absence of components from primate serum or plasma as determined by reverse transcriptase polymerase chain reaction. The titer can also be from about 10^5 to about 10^6 genomes per milliliter of culture medium.

A method for propagating hepatitis C virus in cell culture is also provided, comprising the steps of: (a) contacting a suitable uninfected cell culture with

hepatitis C virus; (b) incubating the contacted cell culture in cell culture medium under conditions to permit infection of the culture cells by hepatitis C virus; and (c) propagating hepatitis C virus in the infected culture cells under conditions to produce a virus titer of at least 10^4 genomes per milliliter culture medium.

A method of diagnosing hepatitis C virus infection in a subject is provided. The method comprises propagating hepatitis C virus from a suspected virus-containing sample from the subject according to the method above and detecting the presence of hepatitis C virus, the presence of hepatitis C virus in cell culture indicating hepatitis C virus infection.

A hepatitis C virus-propagating cell culture propagating hepatitis C virus at a titer of at least 10^4 genomes per milliliter of culture medium is also provided. The virus can also be propagated at a titer of from about 10^5 to about 10^6 genomes per milliliter of cell culture supernatant medium.

The invention further provides a method of screening a compound for antiviral activity, comprising the steps of: (a) contacting the hepatitis C virus-propagating cell culture of claim 12 with the compound; (b) determining the antiviral activity of the compound against the hepatitis C virus propagated by the cell culture; and (c) selecting those compounds having antiviral activity.

DETAILED DESCRIPTION OF THE INVENTION

Virus

The invention provides a composition comprising hepatitis C virus in cell culture supernatant medium at a titer of at least 10^4 genomes per milliliter of culture supernatant medium in the absence of components from

primate serum or plasma as determined by RNA polymerase chain reaction. In the composition of then invention the titer in the cell culture supernatant medium can be from about 10^5 to about 10^6 genomes per milliliter of cell culture supernatant. Titers of up to 10^7 and above may be achieved by routine optimization of the present method. The titer of the HCV of the invention is determined by reverse transcriptase polymerase chain reaction amplification of the viral RNA (RT-PCR or RNA PCR) in the cell culture supernatant as described below.

Other methods of measuring viral propagation can be used and the titer of virus so determined can be standardized to the RT-PCR method to obtain comparable measurements. Such virus measuring methods can include RNA hybridization, plaque assay (Gould, EA and Clegg, JCS Virology: A practical Approach. 1st Ed. Ed: Mahy, BWJ. *IRL Press LTD*, Oxford 43-78, 1985), radio immunofocal assay (Lemon et al. *J. Clin. Microbiol.* 17:834-839, 1983), ELISA (Harlow & Lane *Antibodies; A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1988), radioimmunoassays and hemagglutination tests (Gould and Clegg, 1985). Other nucleic acid amplification methods, such as ligase chain reaction, transcription-based amplification or replicase amplification can be used as described in the art (Wolcott, MJ *Clin. Micro.* Rev. 5:370-386, 1992).

HCV Propagation

A method for propagating hepatitis C virus in cell culture is also provided. The method includes the following steps: (a) contacting a suitable uninfected cell culture with hepatitis C virus; (b) incubating the contacted cell culture in cell culture medium under conditions to permit infection of the culture cells by hepatitis C virus; and (c) propagating hepatitis C virus in the infected culture cells under conditions to produce

a virus titer of at least 10^4 genomes per milliliter of cell culture supernatant medium.

The suitable cell culture can comprise the following cell lines: PK(15) (ATCC CCL 33, porcine kidney, pestivirus growth), NCTC 1469 (ATCC CCL 9.1, mouse liver) and Vero (ATCC CCL 81, African green monkey kidney, flavivirus growth) as shown in the Examples (American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, USA). Other suitable cell lines for use in the present HCV-propagating cell culture method can be selected, as described in the Examples, based on their ability to support growth of other Family Flaviviridae members (pesti- and flavi-viruses) or derivation from liver tissue, the primary target of HCV. A suitable cell line for use in this method must be capable of surviving infection by HCV. Other cell lines can be assessed for suitability for HCV propagation by following the propagation steps taught herein, followed by titering the cell culture supernatant to determine if the cell culture is capable of propagating HCV to a titer of at least 10^4.

The contacting step preferably takes place at from about 0° to about 4° celsius and lasts about one hour. The method of the invention can further include the step of purifying the hepatitis C virus on a sucrose gradient prior to the contacting step as described in the Examples. The HCV used in this method can be obtained from plasma or serum from an HCV infected subject (e.g. human or chimpanzee). This partial purification step can reduce toxicity to the culture cells. The incubating step of the method preferably lasts about eight days, but can last from about six to about nine days. The incubating step preferably takes place at about 37° celsius. Other suitable temperatures can be determined routinely by varying the temperature parameter in the methods described herein.

The present method may include serial passaging, comprising contacting a suitable uninfected cell culture with the hepatitis C virus obtained from step c above. The serial passaging can be repeated at least 6 times and up to 30 times or more. An example of such a method of propagating HCV according to the present invention is provided in the Examples.

A hepatitis C virus-propagating cell culture, propagating hepatitis C virus at a titer of at least 10^4 genomes per milliliter of cell culture supernatant medium is also provided. The HCV-propagating cell culture is obtained according to the methods taught herein. As with the HCV propagation method described above, the cell culture can comprise any suitable cell line. An HCV-propagating cell culture, wherein the virus is propagated at a titer of from about 10^5 to about 10^6 genomes per milliliter of culture medium is also provided.

Diagnosis by Cell Culture

A method of diagnosing hepatitis C virus infection in a subject is also provided. The diagnostic method can comprise the steps of propagating hepatitis C virus from a suspected virus-containing sample from the subject according to the cell culture propagation method described herein and detecting the presence of hepatitis C virus, the presence of hepatitis C virus in cell culture indicating hepatitis C virus infection. The detecting step can comprise performing RT-PCR on cell culture supernatant to determine the presence of HCV RNA, the presence of HCV RNA in the cell culture medium indicating the presence of HCV. Other methods for detecting the presence of HCV include the titering methods described above. Additionally, detection of radiolabeled viral RNA or immunoprecipitation of viral proteins and carbohydrates followed by gel electrophoresis can also be used to detect HCV. Fluorescent antibody detection of viral proteins in

cells (see, e.g., Krawczynski et al. *Gastroenterology* 103:622-629, 1992), western blotting of cell proteins, electron microscopy immune electron microscopy (see, e.g., Bradley et al. *J. Gen. Virol.* 69:731-738, 1988) and *in situ* hybridization (see, e.g., Negro et al. *Proc. Natl. Acad. Sci. USA* 89:2247-2257, 1992) as well known in the art or provided in the Examples can also be used to detect the presence of HCV.

Antiviral Compound Screening

Having provided an HCV-propagating cell culture, a method of screening a compound for antiviral activity is also provided. The screening method can comprise the following steps: (a) contacting the hepatitis C virus-propagating cell culture of the invention with the compound; (b) determining the antiviral activity of the compound against the hepatitis C virus propagated by the cell culture; and (c) selecting those compounds having antiviral activity. In the screening method the compound can be an antibody or other molecule, including synthetic organic or naturally produced molecules (Baron et al. *Microb. Pathog* 7:237-247, 1989; DeClercq, Erik *Antiviral Res.* 12:1-20, 1989). Such organic molecules can have active site-directed properties that inhibit virus specific enzymes *in vitro* or *in vivo*.

Other potential antiviral compounds can include antisense oligonucleotides that are known or shown to inhibit gene expression and are known to be active against many viruses (Miller, PS *Biotechnology* 9:358-366, 1991; Cowsert et al. *Antimicrob. Agents and Chemotherapy* 37:171-177, 1993). Other anti-viral compounds can include anti-idiotype antibodies (raised against neutralizing antibodies), which can block HCV attachment to cellular receptors. Additionally, any molecule that interferes with any phase of the HCV life cycle can be identified and screened according to the present methods.

The step of determining antiviral activity can be a plaque assay as described in the Examples. Alternatively, the step of determining antiviral activity can be a radio-immunofocal assay. Blotting and hybridization to HCV RNA extracted from cells propagating the virus and contacted with the potential antiviral compound can also be used to assess antiviral activity. When the antiviral compound is an enzyme inhibitor, an assay for a virus-specific enzyme activity in HCV-propagating cell culture lysates can be used to determine the antiviral activity of the compound. Alternatively, an uninfected cell culture can be contacted with the compound and the antiviral activity can be determined based on the ability of the compound to inhibit subsequent virus propagation.

Antigen

Purified conformationally correct antigenic polypeptides encoded by the HCV of the present invention are also contemplated. As used herein, "purified" means the antigen is sufficiently free of contaminants (including primate serum or plasma, or viral or cell components with which the antigen normally occurs) to distinguish the antigen from the contaminants or components. The purified HCV antigen and antigenic polypeptides of the present invention are also referred to herein as "the antigen" or "the HCV antigen."

An antigenic polypeptide of the virus can be isolated from the whole virus by chemical or mechanical disruption. The purified fragments thus obtained can be tested to determine their antigenicity and specificity by standard methods. Antigenic fragments generated during the course of cell culture propagation of HCV can also be purified from the supernatant of HCV-propagating culture cells. An immunoreactive fragment is defined as an amino acid sequence of at least about 5 consecutive amino acids derived from the amino acid sequence of the antigen.

The cell culture propagation method, cell culture and high titer virus produced thereby efficiently generate intact virus particles, subunits or other virus-encoded antigens, that when purified, yield large amounts of conformationally-correct viral antigen, either structural or non-structural. These viral antigens (proteins) allow detection of additional immune responses that may not be detected by the current commercial serological assays (especially E1, and E2 (Kuo et al., 1989; and Mimms et al., 1990)) that utilize recombinantly-expressed and denatured (non-native) proteins. Conformationally-dependent antibody(s) can be detected that are prognostic for disease outcome (e.g., chronic vs. convalescent).

Purified Antibodies

A purified monoclonal antibody specifically reactive with native hepatitis C virus or a conformationally correct, immunogenic viral polypeptide encoded by hepatitis C virus, excluding antibodies specifically reactive with recombinant DNA-expressed hepatitis C virus proteins or synthetic hepatitis C virus peptides is also provided. Monoclonal or non-primate polyclonal antibodies raised against native viral antigens (e.g., derived from the present intact virus purified from cell culture or viral-encoded proteins purified from infected cell lysates) are used as diagnostic reagents to detect virus-specific antigens in tissue, or body fluids, and to purify HCV antigens and virions through the use of affinity-capture techniques.

The antibodies can be specifically reactive with only a unique epitope of HCV or they can also react with epitopes of other organisms. The term "reactive" means capable of binding or otherwise associating nonrandomly with an antigen. "Specifically reactive" as used herein describes an antibody or other ligand that does not cross-react substantially with any antigen other than the one

specified, in this case, the HCV antigen. Antibodies can be made as described in the Examples (see also, Harlow and Lane, 1988). Briefly, purified antigen can be injected into an animal in an amount and in intervals sufficient to elicit an immune response. Virus can be purified according to the methods taught herein or other standard protein purification methods with such routine modifications as required. For example, virion proteins can be purified using standard methods for purifying related viruses (e.g., hog cholera virus) with appropriate modifications (see, e.g., Laude, H. *Arch. Virol.* 54:41-51, 1977). For the purification of nonstructural proteins, known purification methods that minimize denaturation can be used (see, e.g., Scopes, RK Protein Purification: Principles and Practice. 2nd ed. *Springer-Verlag*, New York. 329 pages, 1987). Because, the methods taught herein can be used to screen hybridomas for HCV monoclonal antibody production, 100% purity of the starting HCV material is not required.

Antibodies can either be purified directly, or spleen cells can be obtained from the animal. The cells are then fused with an immortal cell line and screened for antibody secretion. Likewise, purified non-primate polyclonal antibodies specifically reactive with the antigen are within the scope of the present invention. The polyclonal antibody can also be obtained by the standard immunization and purification protocols (Harlow and Lane, 1988).

The antibody can be bound to a substrate or labeled with a detectable moiety or both bound and labeled. The detectable moieties contemplated with the composition of the present invention include fluorescent, enzymatic and radioactive markers or ligands that can be bound by other detectable antibodies.

Cell Culture Detection (Diagnosis)

A method of diagnosing hepatitis C virus infection in a subject, comprising propagating hepatitis C virus from a suspected virus-containing sample from the subject according to the method of the invention and detecting the presence of hepatitis C virus, the presence of hepatitis C virus in cell culture indicating hepatitis C virus infection.

Serological Detection (Diagnosis) Methods

Detecting Antibody with Antigen

One example of the method of detecting antibodies specifically reactive with HCV is performed by contacting an antibody-containing fluid or tissue sample from the subject with an amount of the HCV of the present invention and detecting the reaction of the antibody with the antigen. A specific embodiment of the antibody detecting method of the present invention can be an ELISA (Harlow and Lane, 1988). Briefly, purified HCV or HCV from cell culture lysates is bound to a substrate (e.g., membrane, bead, plate); nonspecific proteins are blocked with a suitable blocking agent and then contacted with an unknown sample from the subject for HCV antibody capture by HCV antigen. A secondary antibody is then added which binds to the antibody captured by the HCV antigen. The secondary antibody can include an enzyme moiety which can then be detected by adding the appropriate enzyme substrate. (See, e.g., Harlow and Lane, 1988).

Detecting Antigen with Antibody/Ligand

One example of the method of detecting the antigen is performed by contacting a fluid or tissue sample from the subject with an amount of a purified antibody of the present invention and detecting the reaction of the antibody with the antigen. It is contemplated that the antigen will be on intact virus or will be a conformationally correct polypeptide encoded by

HCV. As contemplated herein, the antibody includes any ligand which binds the antigen, for example, an intact antibody, a fragment of an antibody or another reagent that has reactivity with the antigen. The fluid sample of this method can comprise any body fluid which would contain the antigen or a cell containing the antigen, such as blood, plasma, serum, saliva and urine. Other possible examples of body fluids include sputum, mucus, gastric juice and the like.

The following examples are intended to illustrate, but not limit, the invention. While they are typical of those that might be used, other procedures known to those skilled in the art may be alternatively employed.

EXAMPLES

Cell Culture of HCV

Media

Minimal Essential Media (MEM) is purchased from Gibco (1x liquid with Earle's salts and 1500 mg/l sodium bicarbonate without L-glutamine). Complete MEM contains 2mM L-glutamine, 0.1mM nonessential amino acids, 100U/ml penicillin G, 100 µg/ml streptomycin, 0.075% sodium bicarbonate, and 100mM Hepes buffer. Maintenance media is complete MEM with 2% fetal bovine serum (FBS). Growth media is complete MEM plus 10% FBS.

Sucrose Gradient Purification of HCV

Prior attempts to culture HCV showed that undiluted chimpanzee sera or plasma was very toxic for cell lines. Inoculation with unfractionated (whole) plasma usually led to formation of a gel over the cell monolayer which often resulted in cell-rounding and detachment. Therefore, the virus was partially purified in order to obtain 1) an

inoculum that was less toxic to the chosen cell lines, 2) good recovery of infectious virus. Data determining the buoyant density of the virus by chimpanzee bioassay had delineated conditions for fractionating plasma and recovering infectivity. Partial purification of HCV was therefore performed utilizing these conditions which were subsequently published (Bradley et al. *J. Med. Virol.* 34:206-208, 1991). Briefly, chimpanzee plasma (3ml) was layered onto a 36ml preformed 20-55% sucrose gradient prepared in 10mM Tris-HCl, 1mM EDTA, 150mM NaCl, pH 8.0 buffer. Samples were centrifuged in a Beckman SW27 rotor at 110,000Xg for 22 hr at 5°C. Fractions (1.2ml) were collected and pooled from approximately 21.4%-26.4% sucrose for use as inocula. The HCV-positivity of infected samples was confirmed before and after fractionation utilizing RT-PCR (usually fractionation reduced the titer by a factor of 10 or less). CH810 (1/23/81) was used as an uninfected control. CH771 (10^5 CID/ml plasma pool), and later, PNF 2161 (HCV-positive human plasma, 8/89) were utilized as HCV-infected samples for propagation experiments.

Cell Lines

Cell lines were chosen based on their ability to support growth of other Family Flaviviridae members (pesti- and flavi-viruses) or derivation from liver tissue, the primary target of HCV. Cell lines initially used included NCTC 1469 (ATCC CCL 9.1, mouse liver), Vero (ATCC CCL 81, African green monkey kidney, flavivirus growth), Hep G2 (ATCC HB 8065, human carcinoma), LLC-MK_2 (ATCC CCL 7, rhesus monkey kidney, flavivirus growth), PK(15) (ATCC CCL 33, porcine kidney, pestivirus growth), and Chang liver (ATCC CCL 13, human liver).

Three (PK(15), Vero, and NCTC) showed the greatest promise for propagation based on RT-PCR data, the primary screen for propagation (protocol described below). High

titer production of HCV (>10^4 genome copies/ml) was achieved in both PK(15) and Vero cells. NCTC cells may also support the high titer production of HCV if utilized in the final experimental protocol described below.

Method of Determining Final Protocol

Initial experiments suggested that sucrose gradient-fractionated virus should be incubated for 1 hr (versus 3hr or 6hr) with cells prior to its removal (fractionated virus could still be toxic for cells). In addition, incubation of virus and cells at 4°C during this time (in order to synchronize viral attachment) also appeared to increase the frequency of positive RT-PCR signals. Passing virus-inoculated cells at 1, 2, and 3 weeks indicated that, of the three times, 1 week was most likely to give positive RT-PCR results. Using the above protocol RT-PCR screening indicated that replication may occur within the first 4-5 weeks although with little increase in titer (usually lost with serial dilution) and in many cases disappeared by 10-12 weeks.

Subsequent experiments included Vero and PK(15) cell lines with both CH771 and PNF 2161 as inocula. Both inocula propagated HCV to high titer (>10^4 genomes/ml) in both cell lines. This indicates that the process is not inocula-dependent and suggests that the final high titer virus stocks may be amenable to propagation in other cell lines, as yet, untested (such as NCTC 1469) and that other inocula can be propagated with this method.

Fifty to eighty percent confluent cell monolayers (grown on complete MEM/10% FBS in T-75 flasks) were cooled to 4°C for 45 min. prior to inoculation. They were then washed with 10 ml of incomplete MEM media (4°C) and 15 ml of incomplete MEM (4°C) was added. Sucrose gradient-fractionated virus inoculum (750 µl) was then added and contacted for 1 hr at 4°C. The inoculum was removed and

discarded and 20 ml of room temperature complete MEM/2% FBS was added. Flasks were incubated at 37°C, 5% CO_2 and the virus contacting and incubation steps were repeated with uninfected culture cells (passaged) every 7 days.

One to two days prior to the virus passage, T75 flasks of fresh PK(15) cells in growth media were prepared. On day 7, the growth media was removed from the uninfected flasks and replaced with 17 ml of maintenance media. The media from the infected cells was removed and centrifuged to remove any dead or detached cells. While the media was being centrifuged, the infected cells were scraped from the flask. Centrifuged, infected media (12ml) was added to each infected flask and, through repeated pipetting, a homogeneous cell suspension was created. Each new flask was infected by addition of 3ml of the infected cell/media suspension to the existing 17ml of maintenance media. Flasks were incubated for 7 days at 37°C in 5% CO_2 before repeating the passage. Extra HCV-infected media was stored by adding 1/2 volume of FBS, gently mixing, and freezing at -70°C.

Passages 1 through 8 were performed every 7 days. Based on PCR data suggesting that 8 or 9 days may be the optimal time for passage, passage 9 and subsequent passages were performed after 8 days. RT-PCR data indicated that this change resulted in a significant increase (several orders of magnitude) in viral titer over the next several weeks.

Passaging of cells along with media was discontinued after passage 25. Hybridization data indicated that passaging of supernatant medium without cells may have contributed to a large reduction in virus titer. Cells were therefore passaged with media in all new inoculations. Propagation of frozen virus stock (frozen HCV-containing cell culture medium with cells centrifuged

out) may require passaging at least 2-3 times to increase the titer to levels described in this protocol (see below). Serial dilutions of the day 8 media indicates that the titer reaches 10^5-10^6 detectable genome copies/ml. This titer is greater than that of the original inoculum (Don, #771, chronic phase plasma pool) even after an approximately 10^{-24} dilution (through repeated, serial passage), making it highly unlikely that this HCV is due to carry-over from the original inoculum.

Passaging of infected cells from this original experiment was discontinued following passage 32. Cells and media were frozen back for subsequent use.

Protocol for Cell Culture Propagation of HCV

Propagation of Frozen Virus Stock: The growth media from a T75 flask, containing a fresh 50-80% monolayer of PK(15) cells, is removed and replaced with 17ml of complete MEM (NO serum because the virus is stored in 30% serum). Inoculate (contact) each T75 flask containing culture cells with 3ml of thawed virus stock derived from chimpanzee #771 (10^5 CID/ml plasma pool) or from chimpanzee PNF 2161 (HCV-positive human plasma, 8/89)) and incubate for 8 days at 37°C in 5% CO_2. On day 6 or 7 prepare flasks of fresh PK(15) cells in growth media for use in passaging the virus. On day 8, remove the growth media from the uninfected flasks and replace with 17ml of maintenance media (only the initial contacting step needs no additional serum). Remove the media from the infected cells and centrifuge to remove any dead or detached cells. While centrifuging the media, scrape the infected cells from the flask. Add 12 ml of centrifuged, virus-containing media to each infected flask and, through repeated pipetting, create a homogeneous cell suspension. Infect each new flask by addition of 3ml of the infected cell/media suspension to the existing 17ml of maintenance media. Incubate for 8 days at 37°C in 5% CO_2 and repeat as

above. Any extra HCV-infected media is stored by adding 1/2 volume of FBS, gently mixing, and freezing at -70°C.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Detection of HCV

RT-PCR (RNA PCR) was utilized to detect HCV RNA as the primary screen for the successful propagation of HCV. HCV RT-PCR was performed as described (Beach et al. *J. Med, Virol.* 36:226-237, 1992) using primers from either NS3 or the 5'-untranslated region (5'-UTR)(Alter et al. *New Eng. J. of Med.* 327:1899-1895, December 31, 1992). Briefly, fifty µl of serum or plasma were diluted with 200 µl of 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM ethylene-diamine-tetraacetic acid (EDTA), 0.5% sodium dodecyl sulfate (SDS), 1 mg/ml proteinase K, 30 µg/ml glycogen, and incubated at 37°C for 90 min. The solution was extracted once with an equal volume of Tris-HCl, pH 8.0-saturated phenol, once with phenol: $CHCl_3$, and finally precipitated with ethanol. First-strand cDNA synthesis was carried out by combining the entire RNA extract with 1 µg of random primers (Promega, Madison, WI), heating at 95°C for 2 min, and cooling slowly to room temperature. The RNA/primer solution was combined with 20 units of RNase inhibitor (Boehringer Mannheim, Indianapolis, IN), 12.5 units of AMV reverse transcriptase (BMB), dNTP's to 2.5 mM/nucleotide, and 2 Ml of 10xRT buffer (500 mM Tris-HClpH 8.3, 60 mM $MgCl_2$, 100 mM dithiothreitol, 1,000 mM NaCl) in a final volume of 20 µl. The samples were incubated at 42°C for 60 min, 95°C for 10 min, and then frozen immediately at -70°C. Polymerase chain reaction (PCR) was carried out according to the manufacturer's instructions for Ampli-Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT) in a 10 µl volume utilizing 16.5% of the original RNA extract. The sequence of Choo et al. [1991] was used to design primers that were synthesized using an Applied Biosystems 380B or 391 DNA Synthesizer in the Biotechnology Core Facility Branch of the National Center

for Infectious Diseases at CDC (Atlanta, GA). The first 30-cycle amplification with the external primers (F4' = 5'-CTGCGGGGGCGAGACTGGTTGTGCT-3' [nt4331-4357]) (SEQ ID NO:1), R4' = 5'-GCCGGTATAGCCCATGAGGGCAT-3' [nt4657-4632] (SEQ ID NO:2)) included a 30-sec denaturation at 94°C, a 30-sec annealing at 40°C, and a 1-min extension at 72°C per cycle with a final 7-min extension at 72°C after 30 cycles were completed. Of this solution, 1% was then amplified for another 30 cycles, as described above, with an internal set of primers (F4 = 5' GGAGGTTGCTCTGTCCACCACCGGAGAG-3' [nt4408-4435] (SEQ ID NO:3), R4 = 5' CGCCGCTGGTCGGGATGACGGACAC-3' [nt4610-4586] (SEQ ID NO:4)). Of the final amplified product, 5% to 15% was electrophoresed and visualized on 2% agarose gels as previously described (Ausubel et al. Current Protocols in Molecular Biology, New York: John Wiley & Sons 1989). Positive PCR signals were designated as those displaying a band visible by ethidium bromide fluorescence. All HCV RNA analyses had known HCV-positive and HCV-negative chimpanzee sera included form the initial extraction step. In addition, all PCR analyses had a DNA-minus control that included all PCR components with the exception of cDNA. Oligonucleotide hybridization to amplified products was performed as described (Ausubel et al., 1989). Titrations were performed by making 10-fold serial dilutions of serum or plasma in H_2O prior to performing the 50 µl extraction described above. Titration of a plasma pool from chimpanzee 910, known to have a titer of 1×10^6 chimpanzee infectious doses (CID)/ml, indicated that our current assay is able to detect as little as 0.5 CID or 10 CID/ml.

Detection of either positive or negative strand was determined utilizing strand-specific, rather than random priming, during cDNA synthesis. Later protocols utilized RNAzol for RNA extraction rather than the routine Proteinase K/SDS RNA extraction and a 55°C annealing temperature during amplification. High-titer material was

sometimes found to be weakly positive following only the external amplification indicating that the titer was higher than usually found in routine serum or plasma samples.

Due to the relatedness and sequence conservation between the 5'-UTR's of HCV and the pestiviruses (Han et al. *Proc. Natl. Acad. Sci. USA* 88:1711-1715, 1991) a control assay was performed to determine whether the RT-PCR procedure could detect the pestivirus, bovine viral diarrheal virus (BVDV), a known contaminant of the fetal calf serum used in tissue culture. This assay demonstrated that primer sets used did not amplify BVDV RNA and that a set of functional BVDV PCR primers did not amplify HCV RNA, therefore, the results were not due to cross-contamination with BVDV.

Dideoxy sequencing according to Sanger et al. of the RT-PCR products showed that HCV RNA was amplified (Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).

Although both positive and negative strand HCV-RNA could easily be detected in the cell culture supernatant media, further examination revealed that most serum and plasma samples (including the 2 inocula) screened were also found to be positive for both strands. This made screening for negative strand as a marker for viral replication useless, as it could be argued that the presence of negative strand was due to carry over from the original inocula. Subsequent publications also documented the presence of negative strand HCV-RNA in host circulation (Fong et al., 1991).

Fixation of Cells or Media for EM examination

Infected cells (Vero and PK(15) cells infected with both inocula) were fixed directly on the surface of the flask by replacing the maintenance media with MEM containing from 0.025 to 2.5% formaldehyde for 10 min at room temperature. Cells were then scraped off the flask, pelleted by centrifugation, and stored at 4°C. Cells were then embedded, sectioned, and examined via electron microscopy.

Double-blind viewing of EM sections revealed that only HCV-infected cells (not controls) exhibited spherical, enveloped, virus-like particles with diameters of 40-50 nm. This would be expected for HCV due to its relationship to the flavi- and pesti-viruses which are documented to fall in this size range. Both cell lines exhibited C-type particles of larger diameter which have been previously reported for PK(15) cells. Pathological features such as crystalline arrays, myelin bodies, and bead-like structures were present in far greater numbers in infected cells than in controls. The pathological features found in HCV-inoculated cells changed over time during each passage (days 3-7) indicating a temporal relationship between pathology and the length of post-infection time.

RNA Hybridization

Cells (Vero and PK(15) cells infected with either CH771 plasma or PNF2161 plasma, partially purified by sucrose gradient as described above) from 1 T75 flask on various days post-infection were scraped into fresh MEM and pelleted. The medium was removed and 1ml of RNAzol was added and thoroughly mixed prior to RNA extraction, and loading on dot blots. Positive signals that are significantly greater than cell controls have been observed. The decreased sensitivity of RNA hybridization (10-50 pg) versus RT-PCR (1 genome copy) supports the

conclusion that HCV is being propagated. The HCV genome would need to be present at the levels suggested by RT-PCR in order to be detected by dot-blot hybridization. In addition, low-level carry over, or external contamination, would not give a positive signal by hybridization but would with RT-PCR.

Screening for Vaccine

The culture system allows delineation of conditions for either a plaque assay or a radio-immunofocal assay (RIFA). Although HCV does not appear to cause cytopathic effect (CPE) in either PK(15) or Vero cells, other suitable cell lines may exhibit CPE. RIFA's are not dependent on the cytopathic nature of the virus and, therefore, may be of use with this system. The development of an efficient, easily interpretable assay for HCV titer (either relative or absolute in terms of genome copies present) allows one then to measure the effects of immunological and chemical reagents on production (replication) of the virus. This significantly increases the number of vaccine candidates that can be evaluated due to the limited number and great expense of maintaining chimpanzees for research.

Plaque Assays

Standard laboratory animals are inoculated with the vaccine candidate in order to elicit an immunological response. The resulting antibodies are screened by standard plaque reduction/neutralization methods (Gould and Clegg, 1985) to determine whether the vaccine candidate elicited HCV-neutralizing antibodies in the host animal.

Although methods are described for Vero cells, they can also be used with other mammalian cell lines showing cpe with HCV infection. The first method is ideal for

assaying large numbers of samples and can easily be adapted from microtiter plates, as described here, to plates with larger cups or dishes if, for example, plaque morphology is under investigation. The titration system utilizes carboxymethylcellulose (CMC). Subsequently, a different procedure is described in which agarose is used and the cells are stained with neutral red. This is particularly useful for studying plaque populations when it is important to maintain virus infectivity.

A. Plaque Assay using Carboxymethylcellulose Overlay

Prepare eight serial 10-fold dilutions of virus in maintenance medium. Starting at the eighth virus dilution and using a Pasteur Pipette, place one drop of virus suspension into a cup of the eighth row of a flat-based microtiter plate. Discard any remaining virus suspension from the pipette and repeat the procedure with the seventh dilution into the equivalent cup of the seventh row. Repeat this procedure with each dilution. Immediately after completion of the plate add one drop of Vero cells using a suspension (4×10^6/ml) prepared in maintenance medium. Incubate the plates at 37°C for 3 h. Then to each cup add two drops of maintenance medium containing 1.5% CMC. Incubate the plates in a sealed box at 37°C. Most alphaviruses produce plaques within 2-3 days whereas the flaviviruses take longer. Examine the plates daily until the characteristics of the viruses are known. When cpe is clearly visible at low-power magnification (using an inverted microscope) remove the supernatant medium from each cup of each plate. Add 2-3 drops of formal-saline, leave for 10 min and then replace this with naphthalene black stain. After 30 min at room temperature wash the plates with tap water. Estimate the infectivity (titer; p.f.u.) of the virus samples by counting the number of plaques produced at each dilution. Titrations should be carried out at least in triplicate to avoid problems arising from small errors made in the titration procedure.

However, when large numbers of samples are being compared, the above technique as described will usually produce satisfactory results.

B. Plaque Assay using Agarose Overlay

Prepare confluent monolayers of cells in either small plastic disposable 25 cm^2 flasks or dishes. Prepare serial 10-fold dilutions of virus in maintenance medium. Remove culture medium and add 0.2 ml of virus inoculum, starting from the highest dilution. Ensure that a film of medium completely covers the cell sheet. Either place the flasks on a rocker or every few minutes rock the flasks by hand. Leave at room temperature for 1 h. Remove the inoculum, preferably with a pipette or an aspirator, then add 5 ml of agarose overlay medium. Ensure that the overlay medium has spread evenly over the monolayer, leave at room temperature for 10 min then incubate at 37°C. Different viruses will require different lengths of time before plaques develop. Determine the optimum incubation time by daily examination of the monolayers, starting from the second day for alphaviruses and the fourth day for flaviviruses. When plaques begin to appear add 2 ml of agarose overlay medium containing 0.02% neutral red. Allow 10 min for the agarose to set and then incubate the flask at 37° C in the dark. Within a few hours living cells will be stained by the neutral red and the plaques will appear clear. Do not expose the plates to light for more than a few seconds if it is intended to collect infectious virus from the plaques. For permanent staining, once the plaques have developed and have been stained with neutral red, add 2-4 ml of 10% formal-saline to the dish. After 30 min at room temperature, remove the formalin and agarose and wash the monolayers with tap water. Estimate the virus titer as plaque forming units per ml (p.f.u./ml) as described above by counting the number of plaques at an appropriate dilution.

C. Plaque Reduction Neutralization Test for Togavirus Antibodies

As before, the method for Vero cells will be described, however, other cell lines are suitable. The microtiter method described provides the titer of the antibody as either a neutralization index or as a dilution endpoint titer, the latter being more accurate. However, it also requires more time and equipment.

Place the serum to be tested at 56°C for 30 min to inactivate some of the complement components and also to destroy non-specific inhibitors. Prepare serial 10-fold dilutions of virus and antibody in maintenance medium. If the antibody titer is thought to be relatively low, make serial 2-fold dilutions of antibody instead of 10-fold dilutions. Place one drop per cup of the highest dilution into row 'H' of the microtiter plate, then the next dilution into row 'G', repeating this procedure with successive dilutions to row 'A'. Put one drop per cup of maintenance medium into rows 11 and 12 then add one drop per cup of the highest antibody dilution to row 10. Repeat with the next dilutions. Incubate the plates at 37°C for 60 min then add 1 drop per cup of Vero cells from a suspension (4 x 10^6 cells/ml0 prepared in maintenance medium. Incubate at 37°C for 3 h then add two drops per cup of maintenance medium containing 1.5% CMC and incubate in a sealed box at 37°C or in a CO_2 incubator if the medium is bicarbonate-buffered. When plaques have formed in the control non-antibody treated rows, remove the supernatant medium and replace with 10% formal-saline for 10 min followed by naphthalene black for 30 min at room temperature. Then wash the plates with tap water.

The antibody neutralization titer can be calculated by either of two methods if this type of checkerboard titration has been performed. The first method estimates the dilution of antibody that reduces the plaque numbers

by 50%. For example, if a 1/1000 dilution reduced the number of plaques from 20 to 10 then the plaque reduction neutralization titer would be 1000. This 50% reduction end-point can be determined by plotting antibody dilution against number of plaques. In the second method, which is less precise, the difference between the virus titer at the highest antibody concentration and the non-antibody treated control indicates the serum neutralization index (SNI).

The ability to identify neutralizing antibodies facilitates the identification of neutralizing epitopes on the surface of the virus which could be utilized in the design of new and potentially more effective vaccine candidates. Monoclonal or non-primate polyclonal antibodies identified in this manner can also be important therapeutic agents (antibodies) that could be administered to individuals who have been exposed to potentially infectious material(s). The finding that antibodies to the nonstructural protein NS1 in the flaviviruses are also protective (Gould et al., 1986) suggests that this approach to immunomodulation or abrogation of disease should also be investigated as another avenue for the development of a vaccine against HCV. Other uses of the tissue culture system will be apparent to those skilled in the art of vaccine development.

Additionally, the HCV tissue culture system provides a means for large-scale production of HCV which can be inactivated, such as by formalin inactivation (Hoke et al. *N.Eng.J.Med.* 319(10):608-614, 1988), heat inactivation or radiation inactivation and used as a "killed" virus vaccine. As noted above, other HCV antigens (non-recombinant proteins purified from infected cell lysates) may also elicit protective responses as was found with the NS1 protein of certain flaviviruses (Schlesinger et al. *J. Virol.* 60:1153-1155, 1986).

Vaccine constructs (peptides or recombinant-expressed proteins) are initially screened using the above-described method without the necessity for use of chimpanzees, the only known animal model for HCV infection. Following this preliminary screening the vaccine candidates remaining are tested in primates to provide information on the *in vivo* response to these reagents.

In vivo vaccine testing

Young adult chimpanzees are used. They are housed in open cages in individual isolation rooms maintained under negative pressure. Phencyclidine hydrochloride or ketamine hydrochloride are used to anesthetize the animals at weekly intervals. Each viral variant or other vaccine candidate is inoculated iv into two chimpanzees, as 1 ml of undiluted virus seed per animal. At weekly intervals through 12 weeks postinoculation, blood samples and 16-gauge percutaneous needle liver biopsy specimens are obtained. An additional and final bleeding is done at 17-18 weeks. Stool samples are collected daily and stored at -20°C.

Sera are assayed for alanine aminotransferase and for anti-HCV. Liver needle biopsy specimens are fixed in 10% Formalin, embedded, sectioned, stained with hematoxylin and eosin by standard methods, and microscopically examined. Every other day stool specimens are thawed and homogenized to 20% extracts in phosphate-buffered saline by mechanical shaking with glass beads. After clarification by centrifugation, the extracts HCV are assayed for HCV antigen in cell cultures, but with the use of normal chimpanzee stool extract as negative control.

A preparation of virulent HCV from infected chimp liver with an infectivity titer of about 10^4 to 10^6 is diluted in phosphate-buffered saline and each chimpanzee given 1 ml iv. Two nonimmunized chimpanzees and two

chimpanzees showing immune responses to inoculation with attenuated HCV variants or other vaccine candidates, are challenged. The protective ability of the vaccine candidate is assessed after challenge from bleedings, liver biopsies, stool collection, and assays as described herein.

Screening for Anti-Virals

As outlined above, the use of the HCV tissue culture system in conjunction with the plaque-reduction/neutralization assay as described above or RIFA (Lemon et al., 1983) permit the large-scale testing and design of antiviral reagents. Currently, costly and scarce chimpanzees (usually 5-7 animals per year per testing facility) are available for the in vivo evaluation of an equal or slightly larger number of candidate anti-viral compounds. In sharp contrast the present in vitro HCV tissue culture system can be used to test several hundred to more than 1,000 anti-virals per year.

Inhibition of plaque or faci formation as described above is one method of determining antiviral activity. Alternatively, blotting and hybridization to HCV RNA extracted from cells grown in the presence of antiviral compounds could also serve as a quantitative measure of the compound's efficacy. Compounds to be tested could include monoclonal or polyclonal antibodies, synthetic or natural chemical reagents, active-site directed small organic molecules that inhibit virus-specific enzymes absolutely required for replication in the host subject (human), and anti-sense nucleic acid molecules. This culture system utilizes assays for specific viral enzymes and enables reagents to be designed and tested that specifically inhibit these viral enzymes (e.g., protease, polymerase, helicase).

Production of intact virus particles and other viral proteins permits the production of monoclonal or polyclonal antibodies that include conformationally dependent antibodies, that is, antibodies to native viral antigens in contrast to recombinant DNA-expressed proteins that may lack critically important epitopes. All antibodies to the virus (other than those elicited by natural infections) have, to date, relied on expression of recombinant proteins that may not mimic the actual conformation of surface-exposed virus epitopes and, therefore, only detect non-linear epitopes (Nowak and Wengler *Virology* 156:127-137, 1987). The present conformationally-dependent antibodies can be effective as therapeutic agents against HCV, including but not restricted to their use in individuals already shown to be chronically infected with the virus.

The present cell culture propagation system is useful for studies of the morphogenetic events of HCV infection, including mechanism(s) involved in receptor binding sites (viral attachment), uptake, fusion, uncoating, replication, translation, post-translation modification, packaging, and release of intact infectious particles. Reagents (chemical or immunological, as described above) are identified with this system that can specifically block one or more of these steps. Reagents so identified can then be screened for antiviral activity in the present method.

Infection of Chimpanzees

PK(15) CH771 inocula-infected media (10ml, passage 30, day 8) was centrifuged to remove dead cells and inoculated into each of 2 chimpanzees (CH1492, CH1426). CH1492 first became RT-PCR positive (5'-UTR primers) 15 days after inoculation and CH1426 became RT-PCR positive 22 days after inoculation. An alanine aminotransferase

(ALT) peak in CH1492, indicating liver damage, from day 8 to day 23 post-inoculation was judged not to be pathologically related to viral hepatitis. As of six months fourteen days post-inoculation seroconversion to the 3 HCV antigens routinely screened for in the Abbott diagnostic test have not been detected. However, EM examination of thin-sectioned chimpanzee liver biopsy specimens obtained 3-5 weeks post-inoculation revealed HCV-induced ultra-structural alterations in hepatocyte cytoplasm (Bradley, DW Advances in Hepatitis Research, Chapter 31; Ed. Chisari *Mason Publishing USA, INC.* New York 268-280, 1984). This suggests that tissue culture-derived HCV has infected the above chimpanzees but 1) may be replicating at such low levels that it's minimal release into circulation has not stimulated an antibody response, 2) may be altered by passaging so that commercial antibody assays do not measure seroconversion or 3) may not cause disease due to attenuation and has not yet elicited an antibody response, although characteristic changes were identified within hepatocytes of HCV-infected chimpanzees. A non-pathogenic HCV (as was generated with hepatitis A virus; Bradley 1984) can be utilized as an live-attenuated vaccine.

Throughout this application various publications are referenced in parentheses. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(G) TELEPHONE: 301/496-7056
(H) TELEFAX: 301/402-0220
(I) TELEX: None

(ii) TITLE OF INVENTION: HEPATITIS C VIRUS CELL PROPAGATION AND RELATED METHODS

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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(viii) ATTORNEY/AGENT INFORMATION:
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(B) REGISTRATION NUMBER: 36,016
(C) REFERENCE/DOCKET NUMBER: 1414.060

(ix) TELECOMMUNICATION INFORMATION:
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(B) TELEFAX: 404/688-9880

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGCGGGGGC GAGACTGGTT GTGCT 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCCGGTATAG CCCATGAGGG CAT 23

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGAGGTTGCT CTGTCCACCA CCGGAGAG 28

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGCCGCTGGT CGGGATGACG GACAC 25

What is claimed is:

1. A composition comprising hepatitis C virus in cell culture supernatant medium at a titer of at least 10^4 genomes per milliliter of cell culture supernatant medium in the absence of components from primate serum or plasma as determined by reverse transcriptase polymerase chain reaction.

2. The composition of claim 1, wherein the titer is from about 10^5 to about 10^6 genomes per milliliter of culture medium.

3. A method for propagating hepatitis C virus in cell culture, comprising the steps of:

a. contacting a suitable uninfected cell culture with hepatitis C virus;

b. incubating the contacted cell culture in cell culture medium under conditions to permit infection of the culture cells by hepatitis C virus; and

c. propagating hepatitis C virus in the infected culture cells under conditions to produce a virus titer of at least 10^4 genomes per milliliter culture medium.

4. The method of claim 3, further comprising contacting an uninfected cell culture with the hepatitis C virus obtained from step c.

5. The method of claim 3, further comprising the step of purifying the hepatitis C virus on a sucrose gradient prior to the contacting step.

6. The method of claim 3, wherein the contacting step lasts about one hour.

7. The method of claim 3, wherein the incubating step lasts from about six to about nine days.

8. The method of claim 7, wherein the incubating step lasts about eight days.

9. The method of claim 3, wherein the incubating step takes place at about four degrees celsius.

10. The method of claim 3, wherein the cell culture comprises PK(15) cells.

11. A method of diagnosing hepatitis C virus infection in a subject, comprising propagating hepatitis C virus from a suspected virus-containing sample from the subject according to the method of claim 3 and detecting the presence of hepatitis C virus, the presence of hepatitis C virus in cell culture indicating hepatitis C virus infection.

12. A hepatitis C virus-propagating cell culture propagating hepatitis C virus at a titer of at least 10^4 genomes per milliliter of culture medium.

13. The hepatitis C virus-propagating cell culture of claim 12, wherein the virus is propagated at a titer of from about 10^5 to about 10^6 genomes per milliliter of cell culture supernatant medium.

14. A method of screening a compound for antiviral activity, comprising the steps of:

a. contacting the hepatitis C virus-propagating cell culture of claim 12 with the compound;

b. determining the antiviral activity of the compound against the hepatitis C virus propagated by the cell culture; and

c. selecting those compounds having antiviral activity.

15. The method of claim 14, wherein the step of determining antiviral activity is a plaque assay.

16. The method of claim 14, wherein the step of determining antiviral activity is a radio-immunofocal assay.

17. A purified monoclonal antibody specifically reactive with a native hepatitis C virus or a conformationally correct immunogenic viral polypeptide encoded by hepatitis C virus, excluding antibodies specifically reactive with recombinant DNA-expressed hepatitis C virus proteins or synthetic hepatitis C virus peptides.

INTERNATIONAL SEARCH REPORT

Inter al Application No
PCT/US 94/04929

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K39/29 C12N7/00 G01N33/576 C12P21/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO,A,91 15574 (SOUTHWEST FOUNDATION FOR BIOMEDICAL RESEARCH) 17 October 1991 see page 3, line 23 - page 4, line 27 see page 6, line 4 - page 9, line 2; table 1 see page 17, line 24 - line 32 see page 18, line 24 - page 20, line 23 ---	1-4,6-9, 11-17 5
X Y	WO,A,82 00205 (BAXTER TRAVENOL LABORATORIES,INC.) 21 January 1982 see page 4, line 16 - page 20, line 29 ---	3,4,6-9, 11-17 5
X Y	WO,A,90 00597 (GENELABS INCORPORATED) 25 January 1990 see page 4, line 3 - page 5, line 14 see page 22, line 1 - line 17 see page 27, line 18 - line 25 --- -/--	17 5

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 September 1994	26 -09- 1994

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Intr. mal Application No
PCT/US 94/04929

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,91 02820 (CHIRON CORPORATION) 7 March 1991	17
A	see page 36, line 34 - page 37, line 21 & EP,A,0 414 475 (CHIRON CORPORATION) cited in the application	14-16
X	WO,A,87 05930 (GENELABS INCORPORATED) 8 October 1987 see page 20, line 10 - page 24, line 19	17
Y	JOURNAL OF MEDICAL VIROLOGY vol. 34 , 1991 pages 206 - 208 BRADLEY ET AL 'HEPATITIS C VIRUS:BUOYANT DENSITY OF THE FACTOR VIII-DERIVED ISOLATE IN SUCROSE' cited in the application see the whole document	5
A	WO,A,90 10060 (SOUTHWEST FOUNDATION FOR BIOMEDICAL RESEARCH) 7 September 1990 cited in the application see page 3, line 7 - page 7, line 25	1,2
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